Protocols to assess coagulation following in vitro infection with hemorrhagic fever viruses

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i. Summary/Abstract

During the course of infection with a hemorrhagic fever virus (HFV), the checks and balances associated with normal coagulation are perturbed resulting in hemorrhage in severe cases and, in some patients, disseminated intravascular coagulopathy (DIC). While many HFVs have animal models that permit the analyses of systemic coagulopathy, animal infection models do not exist for all HFVs and moreover do not always recapitulate the pathology observed in human tissues. Furthermore, molecular analyses of how coagulation is affected are not always straightforward or practical when using ex-vivo animal-derived samples, thus reinforcing the importance of cell culture studies. This chapter highlights procedures utilizing human umbilical vein endothelial cells (HUVECs) as a model system to evaluate components of the intrinsic (prekallikrein (PK), factor XII (FXII), kininogen, and bradykinin (BK)) and extrinsic (Tissue Factor (TF)) systems. Specifically, protocols are included for the generation of a co-culture blood vessel model, plating and infection of HUVEC monolayers and assays designed to measure activation of PK and FXII, cleavage of kininogen, and to measure the expression of TF mRNA and protein.

ii. Key Words

Coagulation, tissue factor, factor XII, FXIIa, HUVEC, bradykinin, kininogen, prekallikrein

1. Introduction

Viral hemorrhagic fever (VHF) results from infection with RNA viruses of the *Arenaviridae*, *Bunyaviridae*, *Filoviridae,* and *Flaviviridae* families, and is characterized by coagulation abnormalities, including hemorrhage (reviewed in (1, 2)). Hemostasis, or the balance between coagulation and fibrinolysis, is critical for maintaining the equilibrium between blood clot formation and wound healing and the inhibition of continued clot growth and dissolution of fibrin clots (reviewed in (3)). Coagulation is divided into two pathways, the intrinsic and extrinsic pathways which converge at the common pathway, encompassing fibrin generation, crosslinking, and fibrinolysis (reviewed in (3)).

There have been numerous studies evaluating the effects of HFVs on components of coagulation and it has become increasingly clear that expression and/or activity of coagulation proteins are influenced by infection. For example, tissue factor (TF), the initiator of the extrinsic coagulation cascade, is upregulated in macrophages following infection of nonhuman primates with the filovirus Ebola virus. Likewise, patients infected with the flavivirus Dengue virus who develop Dengue hemorrhagic fever (DHF) have increased levels of TF in their sera/plasma (4-6). DHF patients also exhibit upregulated, von Willebrand factor antigen (vW: Ag), which facilitates platelet attachment and transports factor VIII(7), and plasminogen activating factor (PAI-1), an inhibitor of fibrinolysis (4). Following infection with the arenavirus Junin virus, Argentine hemorrhagic fever patients, display increased PAI-1, and tissue plasminogen activator (tPA), a serine protease involved in the dissolution of clots (reviewed (8, 9)). Furthermore, research from our laboratory demonstrates that infection with either Hantaan or Andes viruses results in the liberation of bradykinin and increased FXII activity and binding (10), all of which are involved in the intrinsic pathway.

Numerous studies have evaluated coagulation in the course of *in vivo* experiments, or through analysis of clinical samples. While these studies are extremely valuable, they can also be expensive, and are limited by sample availability and existing reagents for assessing various coagulation markers in non-human samples. Therefore, we have studied the effects of HFV infection on the coagulation following endothelial cell infection. This chapter will highlight methods to assess select components of the intrinsic and extrinsic pathways and some of the challenges associated with inactivating samples following HFV infection.

2. Materials for analysis of tissue factor mRNA and protein

Cells for infection and viral stocks

1. Pooled human umbilical vein endothelial cells (HUVECs); no greater than passage 7 and cultured with manufacturer's recommendations (Lonza)
2. HEPES buffered saline (HBSS) (Lonza)

3. Phosphate buffered saline (PBS) (Hyclone)
4. TrypLE Express (Life Technologies)
5. 0.1% gelatin (Millipore)
6. EGM-2 bullet kit (Lonza)
7. Phorbol 12-myristate 13-acetate (PMA) (Sigma)
8. Viral stocks: HFVs and respective media for mock samples

mRNA isolation and qRT-PCR

1. RNA isolation and purification: TRizol-LS and Purelink RNA Mini Kit (Thermo Fischer/Life Technologies)
2. O-Ring 1.5mL microfuge tubes (Sarstedt)
3. Brilliant II qRT-PCR 1-Step Master Mix (Agilent)
4. qRT-PCR assays for tissue factor and TATA-box binding protein(TBP) (Life Technologies assays Hs01076032_m1and Hs00427621_m1, respectively)
5. BioRad 0.2mL PCR strip cap tubes
6. BioRad CFX96 qRT-PCR machine and CFX Manager software

Flow cytometry

1. Infected and/or PMA-treated HUVECs
2. PBS (Hyclone; Thermo Fischer Scientific)
3. 10% neutral buffered formalin (PROTOCOL)
4. Permeabilization buffer: 0.1% Triton X-100 in PBS
5. Blocking buffer: PBS with 5.0% fetal bovine serum
6. Tissue factor antibody (Abcam ab48647)
7. Isotype control antibody (KPL affinity purified goat x rabbit IgG heavy and light)

On-Cell ELISA

1. Infected and/or PMA-treated HUVECs
2. HBSS
3. 10% neutral buffered formalin
4. PBS
5. Carnation powdered skim milk (5% and 1% final concentrations in PBS)
6. Tissue Factor antibody Abcam ab48647
7. Secondary antibody Poly GtxRb HRP (Pierce)
8. SureBlue TMB Solution and TMB Stop Solution (KPL)
9. Crystal Violet (CV) solution (Sigma) diluted 1:10 – 0.2% CV and 2% ethanol (final concentration)
10. Cell culture grade water (Hyclone)
11. 1% sodium dodecyl sulfate (SDS)

Vascular Co-culture Model

1. Human umbilical vein endothelial cells (HUVEC), pulmonary artery smooth muscle cells (PaSMC), EGM-2, SmGM-2, trypsin, trypsin neutralizing solution, and HEPES buffer (Lonza)
2. 0.1 % gelatin
3. Cell culture plates

Analysis of Kininogen Cleavage

1. Human umbilical vein endothelial cells (HUVEC), trypsin, trypsin neutralizing solution, and HEPES buffer (Lonza)
2. Bradykinin EIA kit (Enzo Life Sciences)

3. Purified Plasma Prekallikrein (PK), Single Chain High Molecular Weight Kininogen (HK), and FXII (Enzyme Research Labs).
4. HEPES-Tyrode's Buffer
5. Zinc
6. Lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, and protease inhibitors)
7. Rabbit anti-HK (Abnova Catalog # H00003827)

Measurement of Plasma Prekallikrein and FXII Activation

1. Human umbilical vein endothelial cells (HUVEC), trypsin, trypsin neutralizing solution, and HEPES buffer (Lonza).
2. Bradykinin EIA kit (Enzo Life Sciences).
3. Purified Plasma Prekallikrein, Single Chain High Molecular Weight Kininogen, and FXII (Enzyme Research Labs).
4. HEPES-Tyrode's Buffer
5. Chromogenic substrate S2302

Quantitative analysis of Bradykinin Formation

1. Human umbilical vein endothelial cells (HUVEC), trypsin, trypsin neutralizing solution, and HEPES buffer (Lonza)
2. Bradykinin (BK) EIA kit (Enzo Life Sciences)
3. Purified Plasma Prekallikrein, Single Chain High Molecular Weight Kininogen, and FXII (Enzyme Research Labs).
4. HEPES-Tyrode's Buffer
5. HOE 140 (Sigma Aldrich) and Trandolapril (Sigma Aldrich)

3. Methods for analysis of select components of the extrinsic coagulation cascade

3.1 mRNA analysis

Infection and Trizol-LS inactivation

1. Coat T25 tissue culture-treated flasks with 0.1% gelatin for at least 30minutesand then rinse with water.
2. The number of cells plated is dependent on the length of the infection. For a 5 day infection, plate $5x10^4$HUVEC cells per T25 in 5mL EGM-2, 24 hours prior to infection.
3. On the day of infection, remove media and add 1.5mL EGM-2 to each T25 flask so that infections are allowed to proceed in low volume.
4. Infect cells at an appropriate MOI for one hour with rocking every 15 minutes to allow for adsorption. After 1 hour, the viral inoculum can be removed and cells can be rinsed with 2mL of HBSS. Then, 5mL of new EGM-2 can be added. Alternatively, viral inoculum can be left on the cells and the total volume of media can be brought to 5mL. (*see* **Note 1**)
5. After the desired length of infection, remove supernatant (*see* **Note 2)** and rinse cells gently 1x with 2-3mL of PBS.
6. For Trizol-LS, 0.4mL is required for inactivation of a 10cm2 cell culture plate (*see* **Note 3**). However, to effectively cover the T25 surface, 1.0mL of Trizol-LS is added to the flask and rocked periodically. Following a 10minute inactivation, the Trizol-LS sample is transferred to a microfuge tube containing an O-ring. Additionally, pipette a small amount of Trizol-LS around the O-ring gasket before closing tubes.

RNA isolation

1. Following the manufacturer's instructions for compatibility with Trizol, isolate the RNA using the Pure Link RNA Mini Kit.

2. Elute RNA in 30µL of the RNAse free water provided with kit and analyze concentration and purity using the nanodrop.

qRT-PCR

1. For analysis by qRT-PCR the TF and TBP master mix probe sets cannot be multiplexed due to a reduction in signal. Therefore, separate reactions need to be run with each master mix. To set up reactions, see **Table 1**. (*see* **Notes 4 and 5**)
2. qRT-PCR reaction conditions are as follows:
    i. 50°C – 30minutes
    ii. 95°C– 10minutes
    iii. 95°C– 15seconds
    iv. 60°C– 1minutes
    v. Repeat steps 3-4 for 39 cycles
3. Adjust threshold cutoff values where appropriate and ensure appropriate parameters for HEX and FAM signal standard curves (**Figure 1**).
4. Analyze qRT-PCR by normalizing TF Ct values to the housekeeping gene (TBP) values. (*see* **Note 6**)

3.2 Analysis of TF Expression by flow cytometry

Infection and Cell Staining

1. Coat T75 flasks with 0.1% gelatin as described above.
2. For a 5 day infection, plate $2x10^5$ HUVEC cells per T75 in 10mL EGM-2, 24 hours prior to infection.
3. On the day of infection, remove media and add 5mL of media to each T75.
4. Infect cells as described previously.

5. Cells can be treated with PMA as a positive control for TF induction. 2-4hours prior to harvest, add 25-50nM PMA to the culture media.
6. Remove media, and put PMA in appropriate waste container.
7. Rinse cells 1x with 10mL HBSS and add 2mL of trypsin to each flask. Allow cells to detach at 37°C for 3-5minutes.
8. Neutralize trypsin with 8mL of EGM-2 and centrifuge at 1,000rpm for 5minutes. (*see* **Note 7**)
9. Remove supernatant and discard. Add 3-5mL of PBS to each cell pellet and centrifuge as described above.
10. Remove supernatant and resuspend cell pellet in 1.0mL of neutral buffered formalin (10% formalin).Incubate for 10minutes at room temperature. (*see* **Notes 8-9**)
11. Centrifuge cells and discard formalin in appropriate waste container. Wash cells 1x with 1-2mL of PBS.
12. Remove PBS wash and add 100μl of permeabilization buffer to each pellet and pipette up and down with p1000 tip. Allow cells to permeabilize for 10minutes. (*see* **Note 10**)
13. Add 1mL of PBS to wash and centrifuge.
14. Remove supernatant and add 200μl of blocking buffer. Allow cells to block for 10minutes (can incubate longer if desired). (*see* **Note 11**)
15. Separate cells in blocking buffer into two tubes Sarstedt tubes containing 100μl each. One tube will be for the isotype control while the other is for TF antibody.
16. Add 100μl of primary antibody (diluted at1:50 in blocking buffer for a final concentration of 1:100) or isotype control (containing the same concentration of antibody as the TF primary) to the respective tubes. Allow staining to proceed for 1 hour.
17. Wash cells with 1mL of PBS, centrifuge and remove supernatant.

18. Resuspend pellet in 100µL off of secondary antibody (1:1000) diluted in blocking buffer and incubate for 1 hour.
19. Wash with 1mL of PBS, centrifuge and remove the supernatant.
20. Resuspend pellet in 1mL of 10% formalin and inactivate for 24 hours at room temperature. (*see* **Notes 12 and 13**)

3.3 Tissue Factor On-Cell ELISA

1. Plate 2.0×10^4 cells per well (for 3 day infection) in a 0.1% gelatin coated 96-well plate in 100µl final volume EGM-2.
2. Remove media and replace with 80µl EGM-2 24 hours post plating.
3. Infect wells with HFV of choice.
4. On day of harvest, add PMA (25nM final concentration) for 2-4 hours.
5. Remove media from wells with PMA and place in appropriate waste container with 5% Microchem if infectious agent used on the same plate. Then remove media from infected wells and inactivate in 5% Microchem.
6. Rinse wells 1x with 100µL of PBS. (*see* **Note 14**)
7. Fill all wells with 200 µl of 10% formalin and submerge plate and lid in seal bags containing formalin. Then seal in a secondary bag to prevent leaks. Inactivate for 24hours.
8. Remove formalin from bags and plate and discard in waste container.
9. Rinse wells 1x with 100µL HBSS or PBS.
10. Add 50µL of anti-TF antibody (1:100 made in 1% milk) and incubate for 1 hour.
11. Gently wash cells 5-7x with 200uL HBSS or PBS.
12. Add secondary antibody (GtxRb 1:500 in 1% milk) and incubate for 1 hour.
13. Repeat wash as described in step 11.
14. Incubate 100 µl of room temperature SureBlue TMB solution for 30minutes.(*see* **Note 15**)

15. Add 100 μl of TMB Stop Solution and read at 405nm.
16. After reading remove substrate and stop solution and rinse 2x with 100 μl PBS.
17. Add 100 μl of CV solution and allow cells to stain for 10-20minutes at room temperature.
18. Remove CV solution and gently wash several times with water. Continue washing until water removed is clear.
19. Add 100uL 1% SDS to each well and rock/swirl plate at room temperature to solubilize CV.
20. Read at 590nm
21. Normalize each well using the following formula: *OD405/OD590*. The values of blank wells can then be subtracted from experimental well values. (*see* **Note 16**)

3.4 Vascular Co-culture Model

1. Wash cells grown in flasks three times with HEPES buffer and then add trypsin for 5 minutes and then inactivate the trypsin by adding trypsin neutralizing solution.
2. Place HUVEC and PaSMC in separate conical tubes and centrifuge at 1500 rpm for 5 minutes
3. Remove supernatant, resuspend cell pellets in their respective media, and count the number of cells.
4. To form capillary blood vessels in a 0.5 cm^2 well, mix 5 x 10^4 PaSMC cells with 1 x 10^4 HUVEC cells. (*see* **Note 17**)
5. Add 200 µl of combined cells per well.
6. After 24 hours, replace the media 200 µl of EGM-2 and culture for an additional 48 hours.
7. For longer culturing conditions, change the media every 48 hours.
8. Infect cells with the appropriate multiplicity of infection.

3.5 Analysis of Kininogen Cleavage

1. Plate HUVEC on gelatin coated plates and infect at the desired MOI and length of infection.
2. Remove media and wash cells with HEPES-Tyrode's buffer.

3. Dilute HK to 50 nM in the presence of 1 µM Zn^{2+} HEPES-Tyrode's buffer, added to cells, and incubated at 37°C for 1 hour.
4. Wash cells and incubate with 50 nM of PK and FXII diluted in 8 µM Zn^{2+} HEPES-Tyrode's buffer. (*see* **Note 18**)
5. At the end of 1 hour incubation at 37°C, cells are washed and lysed in NP-40 lysis buffer and examined by western blotting.

3.6 Measurement of Plasma Prekallikrein and FXII Activation

1. Plate HUVEC on gelatin coated plates and infect at the desired MOI and length of infection.
2. Remove media and wash cells with HEPES-Tyrode's buffer.
3. Dilute HK to 20 nM in the presence of 1 µM Zn^{2+} HEPES-Tyrode's buffer add to cells and incubate for 1 hour at 37°C.
4. Wash cells and incubate with 20 nM of PK and FXII and 0.8 mM of S2302 in the presence of 8 µM Zn^{2+}HEPES-Tyrode's buffer for 1 hour at 37°C. (*see* **Note 19**)
5. Determine the hydrolysis of the chromogenic substrate S2302 by taking absorbance readings at 405 nm.

3.7 Quantitative analysis of Bradykinin Formation

1. Plate HUVEC on gelatin coated plates and infect at the desired MOI and length of infection.
2. Pre-treat cells with 1 µM HOE 140 and 5 µM Trandolapril for 30 minutes and throughout the experiment. (*see* **Note 20**)
3. Dilute PK, HK, and FXII to 50 nM in HEPES-Tyrode's buffer containing 8 µM Zn^{2+} for 1 hour at 37°C. (*see* **Note 21**)
4. Remove supernatants and perform BK EIA according to the manufacturer's instructions.

4. Notes

Notes for qRT-PCR

1. If a T0 time point is desired, one may choose to harvest a T25 flask immediately following the addition of input virus or after the 1 hour incubation.
2. Ensure that all inocula and supernatants generated post infection are inactivated with 5.0% Microchem or approved disinfectant to inactivate all infectious agents.
3. For HFVs, check with institutional policies on inactivation of *Flaviviridae*, since they are +sense RNA and pose additional risks. Therefore, RNA isolation and qRT-PCR may have to be performed in containment. However, for – sense viruses this method of inactivation is likely acceptable.
4. All samples, including standard curve samples, should be run in at least duplicate.
5. To avoid cross contamination between PCR strip tubes, use filter tips, clean all surfaces and pipettes and cap each row before moving to next well.
6. TF mRNA expression is highly induced following PMA treatment (as described in flow cytometry section). Therefore, the addition of PMA can be used to ensure induction within the given pool of cells and as a positive control.

Notes for flow cytometry

7. Use rotors with sealed gaskets and only open rotors post centrifugation in a biosafety cabinet to avoid potentially aerosolizing virus.
8. For this step it is critical to use a wide pipette tip (such as p1000) to avoid shearing cells. Formalin should also be added to each pellet individually and resuspend immediately to avoid clumping.
9. If using infected cells, or performing the assay in a containment laboratory, cells can be inactivated for 24hours in formalin and then stained following removal from containment. With this option, prolonged fixation may affect signal. Alternatively, cells can be stained in the containment laboratory and then fixed as described in the current protocol.

10. This protocol will measure total TF expression. If cell-surface only expression is desired one can omit the permeabilization step, however this may require re-optimization of the antibody conditions.
11. Standard blocking/FACs buffer can be replaced with a species-specific serum, which should be the same species as the primary antibody.
12. The 24 hour fixation protocol has been performed on cells prior to staining, with some reduction of signal in the basal population. While fixation after TF staining has not yet been performed it is likely to result in better staining. However, if this approach is desired, it should be optimized with this antigen-antibody prior to infection.
13. As described in the legend of Figure 2, the 24hour formalin inactivation can be omitted if no infectious agents are involved (and experiment is not conducted in containment). However, the 10minute fixation should be maintained.

Notes for on-cell ELISA

14. When washing, buffer must be added slowly down the side of the well rather than applied directly to cells as cells can be easily sloughed off. Removing reagents from wells must also be done by pipetting to remove and not by tapping/flicking plate or using a plate washer.
15. Incubation time may vary depending on level of induction in positive control (PMA-treated) samples. However, the 30 minutes suggested in the protocol is recommended for the detection of basal HUVEC TF levels.
16. The purpose of the CV stain is to account for the number of cells in each well so that the OD405 (TF values) are normalized between wells.

Notes for vascular co-culture model

17. To form capillary blood vessels in larger wells, scale up the cell number according to well size while maintaining the ratio of PaSMC.

18. Human mesenchymal stem cells can also be co-cultured with HUVEC to form *in vitro* capillary blood vessels.

Notes for Analysis of Kininogen Cleavage

19. To examine HK cleavage independent of activated FXII, incubate cells with only PK and HK.

Notes for Measurement of Plasma Prekallikrein and FXII Activation

20. To examine activation of PK independently of FXIIa, incubate cells with only PK and HK.

Notes for Quantitative analysis of Bradykinin Formation

21. Inhibitor treatment is required to prevent degradation of BK and prevent binding of BK to its respective receptor. Failure to treat cells will results in inaccurate measurements of BK levels.
22. To measure BK formation independent of FXIIa, incubate cells with only PK and HK.

5. References

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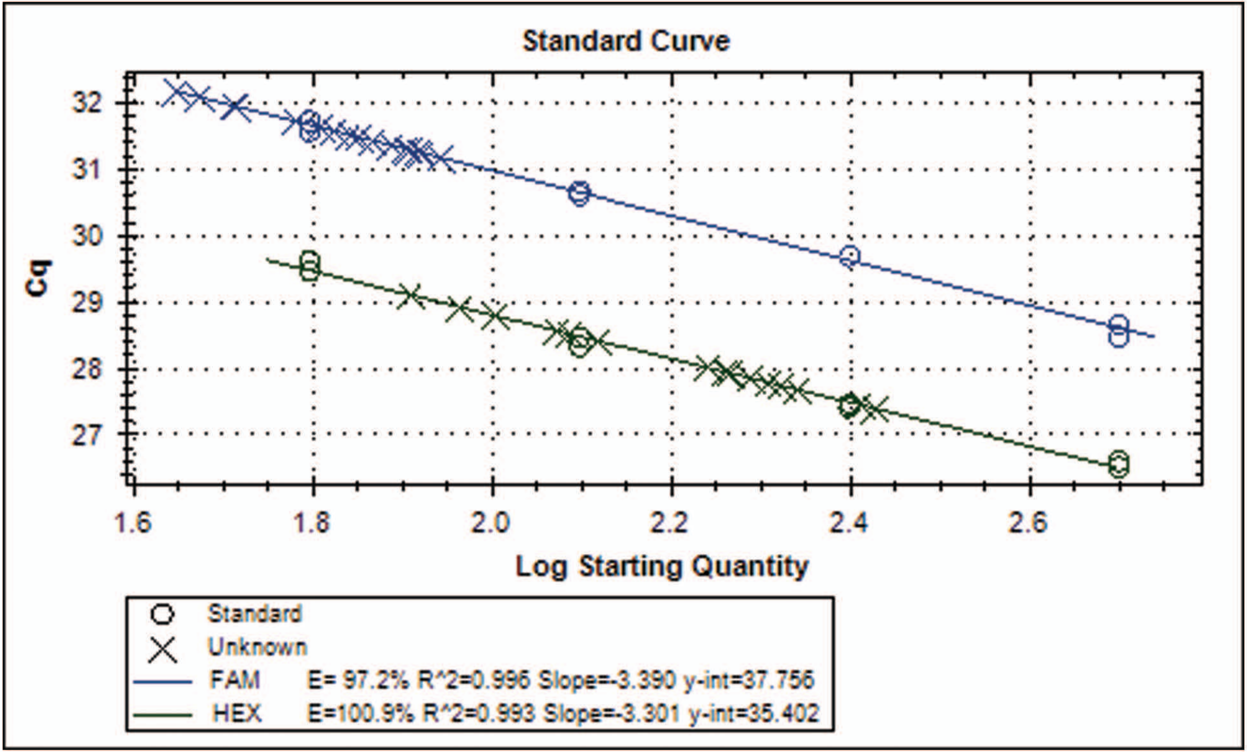
Figure Legends

Figure 1: Standard curves for HEX (TBP) and FAM (TF) signals using HUVEC RNA. Data analyzed using BioRad CFX Manager.

Figure 2: Analysis of TF expression in PMA-treated and untreated HUVECs. HUVECs were treated with 25nM PMA for 4hours. Cells were harvested, fixed (for 10minutes), permeabilized and stained as described in methods with the exception of the 24hr fixation which was not necessary in this experiment since no infectious agents were present.10,000 cells were collected on BD FACS Canto II using FACSDIVA software and then analyzed in FlowJo.



Standard Curve
Cq
32
31
30
29
28
27
1.6
1.8
2.0
2.2
2.4
2.6
Log Starting Quantity
Standard
Unknown
FAM E= 97.2% R^2=0.996 Slope=-3.390 y-int=37.756
HEX E=100.9% R^2=0.993 Slope=-3.301 y-int=35.402

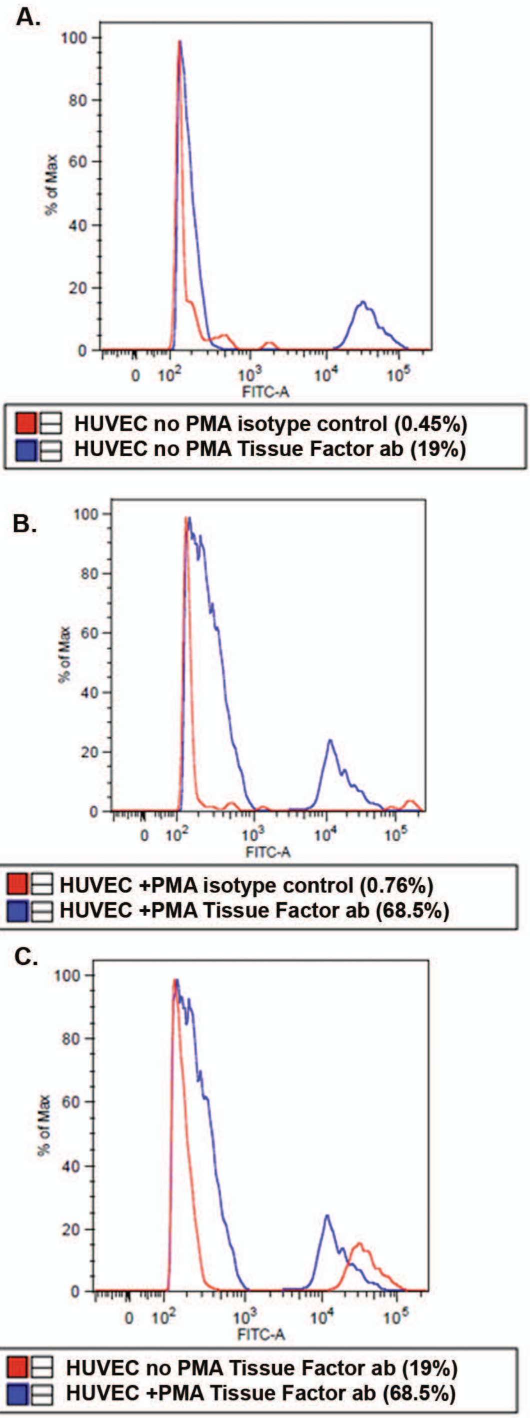

A.
100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
FITC-A
HUVEC no PMA isotype control (0.45%)
HUVEC no PMA Tissue Factor ab (19%)
B.
100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
FITC-A
HUVEC +PMA isotype control (0.76%)
HUVEC +PMA Tissue Factor ab (68.5%)
C.
100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
FITC-A
HUVEC no PMA Tissue Factor ab (19%)
HUVEC +PMA Tissue Factor ab (68.5%)

Table 1: Reaction volumes for TBP and TF qRT-PCR

TBP reaction	(volume in µL)
H2O	6.25
2X Brilliant II qRT-PCR 1-step Master Mix	12.5
20x TBP primer/probe set (final of 0.2x)	0.25
RNA (50ng/uL)	5.00
Reverse Transcriptase/RNAse	1.00

TF reaction	(volume in µL)
H2O	5.25
2X Brilliant II qRT-PCR 1-step Master Mix	12.5
20x TBP primer/probe set (final of 1x)	1.25
RNA (50ng/uL)	5.00
Reverse Transcriptase/RNAse	1.00